[*PSI*$^{+}$] Prion Induction within the Yeast *Saccharomyces* Genera

An undergraduate honors thesis by

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1. ABSTRACT

Prions are self-perpetuating protein aggregates that cause neurodegenerative diseases in mammals and carry heritable traits in yeast. Yeast [*PSI*$^{+}$] prion is the prion isoform of Sup35 protein, an essential translation termination factor. The [*PSI*$^{+}$] prion state can be induced in [*psi*$^{-}$] cells by the overexpression of the full-length Sup35 protein, or only a certain portion of the protein. However, this induction requires the presence of a second yeast prion known as [*PIN*$^{+}$].

While [*PSI*$^{+}$] is present in lab cultures of *S. cerevisiae*, it has not been reported in any other *Saccharomyces* species. In this study, we attempted to induce [*PSI*$^{+}$] in *S. paradoxus* and *S. bayanus*, two close relatives of *S. cerevisiae*. As non-*cerevisiae* species of *Saccharomyces* lack other known prions, we employed a new induction approach based on the overproduction of a chimeric protein composed of portions of Sup35 and Human Progesterone Receptor 6.6 (HPR6.6). This construct can induce [*PSI*$^{+}$] in the absence of other endogenous prions. We next engineered *S. paradoxus* and *S. bayanus* strains with markers that allowed for prion studies in these species. The novel Sup35-HPR6.6 fusion constructs led to [*PSI*$^{+}$] induction in *S. paradoxus*, the sister species of *S. cerevisiae*, but not in the more distantly related *S. bayanus*. We also showed that the prion isoform of Sup35 from *S. paradoxus*, previously known to produce only unstable prions in the heterologous *S. cerevisiae* system, can generate mitotically stable prions in the homologous *S. paradoxus* system. Finally, we propose a model which explains the possible role of the Sup35 and HPR6.6 fusion constructs in [*PIN*$^{+}$]-independent [*PSI*$^{+}$] induction.

2. INTRODUCTION

Prions are a novel type of infectious agents composed entirely of aggregated protein subunits. The apparent lack of nucleic acids-based replication distinguishes prions from all other living or virus-like elements known to date. Instead, prions propagate themselves by converting the normal cellular proteins of the same amino acid sequence to an aggregated form (PRUSINER 1998). These aggregates are characteristic of prion-caused diseases such as bovine spongiform encephalopathy ("mad cow" disease), sheep scrapie, human Creutzfeld-Jakob disease and kuru, and others. In addition, prion-like aggregates are found in several neurodegenerative diseases, including Alzheimer, Parkinson and Huntington diseases (TRZESNIEWSKA *et al.* 2004). Finally, prions are also present in several yeast species (WICKNER *et al.* 2007).

[*PSI*$^{+}$], one of several prions in the yeast *Saccharomyces cerevisiae* (SC), is formed upon the aggregation of the essential protein Sup35. The normal cellular function of Sup35 is termination of translation during protein synthesis. As a result of its dual roles in prion formation and translation termination, Sup 35 has two distinct domains with characterized function: a prion domain at its N-terminus (N domain or Sup35N), and a functional domain that carries out translation termination at the C-terminus (C domain or Sup35C). A third domain is located between the prion and functional domains. However, as its function is not yet known, it is simply referred to as the middle domain (M domain, or Sup35M) (Figure 1).

Saccharomyces paradoxus (SP) and *Saccharomyces bayanus* (SB) are two close relatives of *S. cerevisiae*. The two species diverged from *S. cerevisiae* approximately 5-20 million years ago, with *S. paradoxus* being a closer relative to *S. cerevisiae* than *S.*

bayanus (KAWAHARA and IMANISHI 2007) (Figure 2A). In addition, Sup35 is conserved in the three species, both in terms of overall structure and amino acid sequence (Figure 2B). Despite the overall relatedness of the species, the [*PSI*$^{+}$] prion has not yet been reported in *S. paradoxus* or *S. bayanus*.

In this study, we created *S. paradoxus* and *S. bayanus* strains that allow prion studies in them. We then used a novel construct in an attempt to induce the prion state of Sup35 in *S. paradoxus* and *S. bayanus* and characterized any induced prions based on their stability, curability, and *in vivo* Sup35 solubility/aggregation patterns.

3. LITERATURE REVIEW

3.1. Discovery and Structure of Prions

Scrapie, a wasting disease in sheep, has been known in Europe since the Middle Ages. Veterinary doctors were conducting studies on infected sheep as early as the 1950s. One of the first reviews of the novel heritability patterns of the disease appeared in 1960, but it failed to provide an explanation about the nature of the causative agent (PARRY 1960). Amidst attempts to isolate and characterize the agent, Tikvah Alper proposed the radical idea that the cause of this infectious disease did not contain any nucleic acids. He based his claim upon the apparent high resistance of the infectious particle to ionizing and ultraviolet radiation, agents known to damage DNA and RNA (ALPER *et al.* 1967). Not surprisingly, Apler's proposal was met with vehement outcry from the scientific community. Most researchers maintained their positions that the elusive particle was a slow-acting virus (ADAMS and CASPARY 1967). Despite the mounting evidence to the

contrary, some scientists did not change their views even into the 1990s (BROWN and GAJDUSEK 1991).

The first support for Apler's hypothesis came from a surprising source. A mathematician, J.S. Griffith, used a mathematical proof to show that the scrapie agent could not only be a protein, but could also self-replicate (1967). He even went as far as to suggest that the elusive infectious agent was a normal cellular protein that adopted novel conformations (GRIFFITH 1967). Later, mostly biochemical, research revealed even more secrets of the confusing disease-causing particle. Treatment with nucleases, phospholipases and proteases seemingly did not affect the infectivity of the agent (PRUSINER *et al.* 1980). These results suggested that the agent could be composed of aggregated, highly stress-resistant proteins, which led Prusiner to finally propose the term 'prion' in 1982 as an abbreviation for an '*pro*teinatious *in*fectious particle' (PRUSINER 1982). Soon thereafter researchers were able to find the previously hypothesized protein aggregates in the brains of scrapie-infected animals. Paul Bendheim and team were able to obtain antibodies to the prion protein, which they used to visualize the spread of the aggregates across the brain (BENDHEIM *et al.* 1984). In addition, the aggregates formed highly organized structures called "amyloid fibers" (DEARMOND *et al.* 1985). At about the same time, the prion world was starting to grow as prions were raised as the possible causative agents of bovine spongiform encephalopathy ("mad cow" disease) (HOLT and PHILLIPS 1988), and as explanations for the unexpected heritability patterns seen in yeast (COX *et al.* 1988).

Major advances were being made in elucidating the precise structure and replication of prions as well. Analysis of initial prion aggregation with electron

microscopy and infrared spectroscopy revealed that the amyloid fibers were composed of α-helices and β-sheets (GASSET *et al.* 1992). It was later determined that the α-helices are a transient structure in the process of the conversion of normal cellular proteins into the insoluble, highly organized amyloids composed of β-sheets (PAN *et al.* 1993). The prions then replicated through the formation of infectious nuclei, or seeds, that catalyzed the conversion of the non-prion isoform of the protein into a prion (LANSBURY and CAUGHEY 1995).

3.2. Yeast Prions

As in the case of mammalian prions, yeast prions were known long before their identity and properties were suspected. For example, ψ^+/[*PSI*$^+$] was a long-known extra-chromosomal factor in the yeast *Saccharomyces cerevisiae*. In a 1988 review, Brian Cox, one of the main investigators working with [*PSI*$^+$], summarized the existing evidence pointing to a novel mode of heritability, but did not make a connection with the already known mammalian prions (COX *et al.* 1988). Reed Wickner seems to be the first yeast scientist to notice the connection. While working with [*URE3*], another yeast element with puzzling heritability, he suggested that it has prion-like properties (WICKNER 1994). Later that same year, two independent labs reported in the same issue of *Genetics* that mutations in the *SUP35* gene are responsible for the [*PSI*$^+$] phenotype (DOEL *et al.* 1994), (TER-AVANESYAN *et al.* 1994). These two studies explained that certain mutations can make the protein less likely to form prions.

A variety of factors can influence the presence and transmission of the [*PSI*$^+$] prion. For example, chaperone proteins normally involved in stress responses are

intricately involved in prion induction and transmission. In addition, different chaperone families can have different effects on induction and transmission (NEWNAM *et al.* 1999).

Another important factor is the presence of an additional yeast prion called [*PIN*$^+$], the prion isoform of Rnq1 protein. In the absence of the [*PIN*$^+$] prion, *de novo* [*PSI*$^+$] prion induction is rarely observed (DERKATCH *et al.* 1997), (BORCHSENIUS *et al.* 2002). It is likely that [*PIN*$^+$] interacts with soluble Sup35 to provide a scaffold on which the prion conversion of Sup35 can occur (Figure 3). However, certain amino acid sequences can help overcome the [*PIN*$^+$] requirement (DERKATCH *et al.* 2000).

Finally, a fortunate discovery led to the identification of guanidine hydrochloride (GuHCl), a chemical compound that can cure yeast prions (TUITE *et al.* 1981), and is thus used as a defining feature of whether something is a prion. The mechanism of action of GuHCl probably involves the inactivation of one of the chaperone proteins that are involved in prion propagation (FERREIRA *et al.* 2001).

3.3. Species Barrier in Prion Transmission

In order to facilitate prion research, scientists tried to create a mouse model of prion infections. However, they noticed that transmission of sheep scrapie to mice was decreased as compared to transmission within the same species, giving rise to the so called “species barrier” phenomenon. Lansbury and Caughey tried to explain the existence of the species barrier by proposing that the interacting proteins between different species contained amino acid substitutions that made aggregate formation less favorable (1995). Experiments that have been carried out with yeast prions seemed to support Lansbury and Caughley’s hypothesis: prion proteins from highly divergent

species did not co-aggregate (SANTOSO *et al.* 2000). However, studies with mammalian prions showed that binding was not significantly affected despite the sequence divergence between the interacting proteins, but prion conversion was inhibited (HORIUCHI *et al.* 2000). A recent study carried out in yeast supported the latter theory about the role of aggregation. Specifically, Chen *et al.* showed that proteins from closely-related species co-aggregated, but there was no prion conversion (2007). Thus, they proposed that there could be different mechanisms for the species barrier. While prions from divergent species do not co-aggregate, the species barrier between closely related species is controlled at the level of aggregate conformation instead of binding.

3.4. Prion Strains

The presence of different prion isoforms coming from the same protein and amino acid sequence have been known since the early 1990s. For example, different strains (also called isolates or variants) of mammalian prions have different brain distribution and infection patterns. Still, the pattern of each individual strain is highly reproducible (DEARMOND *et al.* 1993). The same effect is observed in yeast: the same protein can give rise to multiple strains upon conversion into a prion, both for the [*PSI*$^{+}$] (DERKATCH *et al.* 1996) and [*URE3*] prions (SCHLUMPBERGER *et al.* 2001). The likely cause is that various conformations can be formed in the initial steps of prion conversion. Each conformation can then "seed" a different strain of prion proteins (CHIEN and WEISSMAN 2001). Work done by Uptain *et al.* clarifies that the strains also differ in terms of ratios of aggregated to non-aggregated proteins (2001). The authors also suggest that different conformations are more thermodynamically favored than others, which explains the different protein

ratios. The favored conformations of yeast prions can propagate better and are called "strong" strains; the less favored conformations are weaker and harder to transmit and are called "weak" strains.

3.5. Ways to Study Prions

While a large number of techniques are available for the detection and analysis of both mammalian and yeast prions, only a few of them are relevant for this study. These include widely-used methods applied to yeast prions, including detection with a nonsense suppression assay, *de novo* prion induction, and differential centrifugation. The reader is referred to a review by Chernoff *et al.* for a more exhaustive information on several additional methods (2002).

3.5.1. [PSI$^+$] Prion Detection

Because Sup35 is a translation termination protein, factors that decrease its function also increase the read-through of stop codons, including premature ones. The presence of [*PSI*$^+$] in yeast cells could be detected as early as 1983 if the cells contained an *ADE2* allele (part of the adenine biosynthetic pathway) that contained a premature UGA codon (TUITE *et al.* 1983). A number of other alleles have the same effect. For example, the *ade1-14* allele, also involved in the adenine pathway, contains a premature UGA stop codon as well (NEWNAM *et al.* 1999). In [*psi*$^-$], prion-free cells, Sup35 is functional, stops translation at the UGA codon, which results in a truncated Ade1 protein. The truncated version of Ade1 is nonfunctional, making cells unable to grow on media lacking adenine (-Ade media). In addition, a red precursor accumulates in the cells,

giving rise to red colonies when they are grown on YPD (rich yeast medium). However, if Sup35 is in its aggregated [*PSI*$^+$] form, it cannot carry out its function completely and there is a readthrough of the stop codons, allowing the strain to grow on media lacking adenine (CHERNOFF *et al.* 2002). Thus, this suppression of translation termination resulting in growth can be used to detect prions (Figure 4). In this study, we used strains containing only the *ade1-14* allele as a reporter for the presence of prions.

3.5.2. Differential Centrifugation

The aggregation of Sup35 in [*PSI*$^+$] strains changes the solubility of the protein. This change can be detected by a differential centrifugation assay. Sup35 in [*PSI*$^+$] can be pelleted by centrifugation at 12,000 *g*, while Sup35 in [*psi*$^-$] cells remains mostly soluble even at 100,000 *g.* The different solubility patterns can be visualized by performing Western blotting on both the pellets and supernatants after centrifugation with an antibody against Sup35 (PATINO *et al.* 1996).

3.5.3. Prion Induction

One possible way to introduce prions to cells is to infect the cells, for example by mating [*psi*$^-$] haploids to [*PSI*$^+$] strains. Still, it is also possible to induce *de novo* prion formation by the overexpression of either the full-length prion protein (DERKATCH *et al.* 1996), or only portions that include the prion domain (GLOVER *et al.* 1997), (BORCHSENIUS *et al.* 2002). However, induction of [*PSI*$^+$] using both of these methods requires the presence of the [*PIN*$^+$] (BORCHSENIUS *et al.* 2002). The [*PIN*$^+$] requirement can be overcome by the addition of a short extension of 16 amino acids to Sup35NM

(DERKATCH *et al.* 2000). Another extension with the same effect is a portion of the Human Progesterone Receptor protein 6.6 (HPR6.6). The Sup35NM-HPR6.6 fusion construct efficiently induces [*PSI*$^+$] in a [*PIN*$^+$]-independent manner (Chernoff lab, unpublished data).

In this study, we used the above-mentioned construct as an inducer for [*PSI*$^+$]. In particular, Sup35NM from *S. cerevisiae*, *S. paradoxus* and *S. bayanus* were used to create three possible inducers: Sup35NM$_{SC}$-HPR6.6, Sup35NM$_{SP}$-HPR6.6 and Sup35NM$_{SB}$-HPR6.6. The inducers were on plasmids under the *CUP1* copper-inducible promoter, allowing for their overexpression. Each of the three plasmids was transformed into each of the three species. Furthermore, *S. cerevisiae* and *S. bayanus* contained chromosomal *sup35* deletions, and the essential gene was replaced by plasmids containing the full-length *SUP35* gene from one of the three species. Thus, even within one species, there were nine combinations of an HPR6.6-fusion construct and full-length protein. The *S. paradoxus* strain used for this study contained its wild-type copy of *SUP35*, but was still transformed with the three inducers.

3.6. Implications of Current Study

The findings from the study show whether the cell environment or the intrinsic properties of the Sup35 protein are responsible for its prion-forming abilities. Furthermore, because the inducers and full-length proteins will be from different species, the study represents another test for the model that was proposed to explain the species barrier for closely-related species.

4. MATERIALS AND METHODS

4.1. Genetic and Microbiological Techniques

4.1.1. Yeast Strains and Maintenance

Standard growth media were used for the maintenance of yeast strains as described previously (SHERMAN 2002). As needed, additional chemicals were added at the concentrations indicated below.

All *S. cerevisiae* strains were isolates of the strain GT671 (*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN LEU2 SUP35SC*] [psi^-][pin^-]), but containing different plasmids (Table 1). *S. cerevisiae* strains were grown and incubated at 30ºC unless otherwise specified (e.g, heat shock).

All *S. paradoxus* strains were derived from the strain GT983-2A (*MATa ura3-P2 lys2 Δho::KanMX6* [psi^-][pin^-]), originally derived from SP7-1D (CHEN *et al.* 2007). All *S. bayanus* strains are derivatives of Su1A (*MATa ura3-1 ho::KanMX4*) (TALAREK *et al.* 2004) *S. paradoxus* and *S. bayanus* strains were maintained and manipulated at 25ºC unless otherwise specified.

Information about all strains used in this study can be found in Table 1. Table 2 contains descriptions of all primers used in strain creation. In addition, Figure 5 shows a graphic representation of the regions that primers for *ADE1* cover.

4.1.2. Plasmid Creation and Transformation in E. coli

Shuffle plasmids containing both bacterial and yeast genetic markers were created using a Quick Ligation™ Kit (New England Biolabs, Inc. Beverly, MA), following the

manufacturer's protocol, with the exception that the cells were heat shocked at 42ºC, instead of 37ºC. All plasmids used in this study are described in Table 3.

4.1.3. Yeast Genomic DNA Isolation and PCR

In order to isolate their genomic DNA, yeast cells were grown overnight in 10mL of liquid YPD. The cells were then pelleted at 2500rpm for 5 min, resuspended in 0.5mL of 1*M* sorbitol, 0.1*M* NaEDTA (pH 7.5), and transfered to a 1.5mL microcentrifuge tube. 40µL of 4mg/mL solution of lyticase was added, and the cells were incubated overnight in a 37ºC water bath. After pelleting at 3000rpm for 5min, the supernatant was removed, the cells were resuspended in 0.5mL of a 50m*M* Tris-Cl (pH 7.4), 20m*M* NaEDTA solution, and treated with 55µL of 10% SDS. Following incubation at 65ºC for 30min, 0.2mL of 5*M* potassium acetate was added, and the cells were left on ice for one hour. Cell debris was separated from the DNA by high speed centrifugation. The supernatant was transferred to another microcentrifuge tube, and DNA was precipitated with 0.75mL of isopropanol at -20ºC for 30 min. The DNA was later pelleted, washed with 70% ethanol, dried, and resuspended in 0.4mL of a 10m*M* Tris-Cl (pH 7.4), 1m*M* NaEDTA solution (1X TE). Any residual RNA was removed by treatment with 2.2µL of 10mg/mL solution of RNase A at 37ºC for 30 min. The remaining DNA was precipitated with 0.84mL of 95% ethanol at -20ºC for 30 min. Finally, the DNA was collected, washed, dried and resuspended in 50µL of 1X TE.

When needed, approximately 100ng of the genomic DNA was used for each PCR sample. The samples also contained 5µL of ThermoPol Buffer, 4µL of 10µ*M* dNTPs, 1µL of 50m*M* for each forward and reverse primer, and 1000 units of either Taq or Deep Vent

polymerases. De-ionized water was used to adjust the final volume to 50µL. The reaction was carried our in Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) for 35 cycles and conditions appropriate for each primer set.

4.1.4. Yeast Transformation

To prepare yeast cells for plasmid transformations, the cells were grown overnight in 10mL liquid YPD. An equal volume of YPD was then added, and the cells were allowed to grow for 3-4 more hours to reach OD_{600} of approximately 2. The cells were collected by centrifugation for 5 min at 3000 rpm, washed with 10mL of 1X TE, and resuspended in 10mL of a 100m*M* LiAc, 10m*M* Tris-HCl, 1m*M* EDTA pH8.0 solution. After incubation for 60 minutes, the cells were collected and concentrated by resuspending them in 0.5mL of the same solution. 50µL of yeast cells were used for each transformation reaction. The reactions also contained approximately 20µg of carrier DNA and 1µg of transforming DNA (plasmid or PCR product). After incubating the reactions for 30min, 350µL of a 100m*M* LiAc, 40% PEG 4000, 10m*M* Tris-HCl, 1m*M* EDTA pH8.0 solution was added and the cells were incubated for an additional hour. Finally, the cells were heat shocked at 42ºC for 7 min and plated on the appropriate solid medium.

4.1.5. Yeast Sporulation and Dissection

Yeast cells to be dissected were grown on pre-SPO media containing any amino acids necessary for growth for one day, replica-plated to SPO media with the necessary amino acids, and allowed to grow for five days. A small patch of cells was dissolved in 40µL of 4mg/mL solution of lyticase, mixed well by vortexing, and incubated at 37ºC for

10 min. The cells were collected by centrifugation at 14,000 rpm for 10 min and resuspended in 20μL of water. A small amount of the cell solution was spotted into dissection media and dissected using a Singer MSM 300 micromanipulator according to the manufacturer's instructions.

4.2. Prion Induction by Overexpression of Sup35NM

The *sup35Δ* strains of *S. bayanus* and *S. cerevisiae* and the *SUP35* strain of *S. paradoxus* were transformed with the HPR6.6-fusion constructs described above. 12 individual transformants were checked for each possible combination. The transformants were then replica-plated to media containing 10, 25, 50 or 100μ*M* of $CuSO_4$ to overexpress the inducers. After 1-2 days on copper-containing medium, the transformants were replica-plated to media lacking adenine. If [PSI^+] induction occurred, it could be detected at approximately day 7 of incubation.

4.3. Characterization of Prions

4.3.1. Mitotic Stability of Prions

To check prion stability, colonies containing induced prions were streaked out for single colonies on YPD medium. Each streak-out gave rise to approximately 100-200 mitotic derivatives of each starting colony. Stable prions gave rise to colonies of mostly one color (white or light pink). Unstable prions gave rise to mostly red colonies.

4.3.2. Prion Curability

Prion-containing colonies were spotted on YPD medium containing 50μ*M* GuHCl. The plates were incubated overnight and then a new spot was made on the same plate using the spot made the previous day. The procedure was repeated one more time for overall three passages on GuHCl media. The third spot was streaked out for single colonies, and four colonies from each spot were checked for growth on media lacking adenine. Colonies passed only through YPD were used as a control. Cured colonies did not grow on media lacking adenine.

4.3.3. Protein Isolation and Differential Centrifugation

In order to isolate proteins from yeast cells, the cultures were grown overnight in 10mL YPD. On the next day, the cultures were incubated with 200μg/mL cyclohexamide for 20 min. The cells were collected by centrifugation and washed and resuspended in lysis buffer (25n*M* Tris pH 7.5, 100m*M* NaCl, 1n*M* DTT, 10m*M* EDTA, 2n*M* PMSF, 10% cOmplete Protease Inhibitors Cocktail (Roche Applied Sciences, Indianapolis, IN)). The cells were then lysed by vortexing for 90s with an equal volume of glass beads. Half of the total cell lysate was centrifuged for 15 min at 10,000 *g* in a microcentrifuge for Sup35 protein or 30 min at 53,000 *g* in a Beckman Coulter Optima^{TM} MAX untracentrifuge for Rnq1 protein in order to separate aggregated from non-aggregated proteins. The supernatant, containing the soluble proteins, was moved to another eppendorf tube and the pellet with the aggregated proteins was resuspended in an equal volume of lysis buffer.

4.4. Western Blotting

The total lysate, supernatant, and pellet obtained by differential centrifugation were run on an 8% polyacrylamide separating gel with a 5% stacking gel. The proteins were transferred to a Hybond™ ECL™ nitrocellulose membrane (GE Healthcare, Piscataway, NJ) using a Bio-Rad Trans-Blot® sD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked overnight with a 5% non-fat milk solution, and washed three times with 1X TBS, 0.1% Tween for 15min each. A primary rabbit anti-Sup35NM or anti-Rnq1 antibody was then applied for 90 min at a 1:2000 (v/v) dilution for Sup35 and a 1:5000 (v/v) dilution for Rnq1. After three more washes, the membrane was incubated with a secondary rabbit HRP antibody for 45 min at a 1:2000 (v/v) dilution for Sup35NM and a 1:3000 (v/v) dilution for Rnq1. Finally, following three more washes, the membrane was developed.

5. RESULTS

5.1. Strain creation for *S. paradoxus* and *S. bayanus*

In order to use the nonsense suppression assay for [PSI^+] detection in *S. paradoxus* and *S. bayanus*, the *ade1-14* allele had to be introduced in these two species. The introduction was achieved by first replacing the species' wild-type *ADE1* genes with *URA3* from *S. cerevisiae* (Figure 6A). The Ade^- strains were then transformed with a PCR-amplified $ade1\text{-}14_{SC}$ fragment. For *S. bayanus*, the *ade1-14* fragment contained 40 base pairs on each side of the open reading frame (ORF) to facilitate homologous recombination (Figure 5A). The fragment was then transformed into GT986 (MATa

ura3-1 ade1::URA3$_{SC}$ ho::KanMX4). Potential transformants were screened by growth on synthetic medium containing 1mg/mL of 5-fluoroorotic acid (5-FOA) to select for *URA3* mutants. A total of 24 Colonies showed growth on 5-FOA, and were checked by PCR and sequencing. Two Ura$^-$ potentials had replacement of *URA3* with *ade1-14$_{SC}$* (Figure 6C). After sequencing with a variety of primers (Figure 5A), one of the potentials contained the correct *ade1-14$_{SC}$* sequence, and was saved as GT1038 (MATa *ura3-1 ade1::ade1-14$_{SC}$ ho::KanMX4*).

For the *S. paradoxus* construction, *ade1-14* was first amplified with 40 base pairs on each side of the open reading frame (Figure 6B). A second set of primers used the product of the first PCR as a template to introduce 80 more base pairs homologous specifically to the promoter and terminator regions of *ADE1* in *S. paradoxus* (Figure 5B). The scheme outlined for *S. bayanus* was then followed by transforming the *ade1-14* PCR fragment into GT992 (*MATa ura3-P2 lys2 Δho::KanMX6 ade1$_{SP}$::ade1-14$_{SC}$* [*psi$^-$*][*pin$^-$*]). A total of 22 potentials had the *URA3$_{SC}$::ade1-14$_{SC}$* replacement (Figure 6C), but only five showed actual non-sense suppression in *S. paradoxus*. Two of these five potentials were not sequenced, one showed the correct *ade1-14$_{SC}$* sequence, and two had PCR-generated errors. A strain containing *ade1-14$_{SC}$* with two PCR-generated errors (GT1142: *MATa ura3-P2 lys2 Δho::KanMX6 ade1$_{SP}$::ade1-14$_{SC}$* (G277N, V278I) [*psi$^-$*] [*pin$^-$*]) was used for induction experiments in *S. paradoxus* (Figure 6D).

To obtain the *sup35Δ* strain of *S. bayanus*, a diploid was first obtained by mating GT1020 (MATα *ura3-1 ho::KanMX4 lys2*) and GT1028 (MATa *ura3-1 ade1::ade1-14$_{SC}$ ho::KanMX4*). The diploid was selected on media lacking lysine and uracil, and transformed with a PCR-amplified gene for resistance against the antibiotic ClonNAT.

The diploids were then transformed with a plasmid containing Sup35, sporulated and dissected. All spores were checked for growth on YPD+100mg/mL ClonNAT (from Werner BioAgents, Jena, Germany) and media lacking uracil, lysine or adenine. One spore that did grow on YPD+100mg/mL ClonNAT, but not on media lacking uracil, lysine or adenine, was saved as GT1122-2B (MATα *sup35Δ::ClonNAT ura3-1 lys2 ade1::ade1-14*$_{SC}$). Strains containing Sup35 plasmids from all three species were obtained from this parental strain.

5.2. *S. paradoxus* and *S. bayanus* are [*pin*$^-$]

Because of the importance of [*PIN*$^+$] prion in [*PSI*$^+$] induction, *S. paradoxus* and *S. bayanus* were checked for the presence of [*PIN*$^+$] by differential centrifugation (Figure 7). *S. cerevisiae* strains that were known to be [*PIN*$^+$] or [*pin*$^-$] were used as positive and negative control, respectively. It is clear that all of the Rnq1 protein, the determinant of [*PIN*$^+$], is aggregated and thus found in the pellet of [*PIN*$^+$] strains. In contrast, the great majority of Rnq1 is found in the supernatant of [*pin*$^-$] cells because the protein is still soluble. The distribution of Rnq1 protein in *S. paradoxus* and *S. bayanus* is similar to the distribution in [*pin*$^-$] *S. cerevisiae*: the majority of the protein is soluble and detected in the supernatant (Figure 7). Thus, the *S. paradoxus* and *S. bayanus* strains tested in this assay were both [*pin*$^-$].

5.3. Sup35NM-fusion constructs

Human Progesterone Receptor 6.6 (HPR6.6) is a human nuclear receptor. When part of it is fused to the C-terminus of Sup35NM (Sup35NM-HPR6.6), the construct is

capable of inducing [*PSI*$^+$] in *S. cerevisiae* strains. In order to investigate the possibility that the Sup35NM-HPR6.6 constructs can induce [*PSI*$^+$] in [*pin*$^-$] background of *S. paradoxus* and *S. bayanus* as well, we created expression constructs where the chimeric protein was under the control of the *CUP1* copper-inducible promoter (Figure 8A, B). The fusion proteins were expressed from a shuffle plasmid with *URA3* yeast marker (Figure 8C).

5.4. [*PSI*$^+$] induction in *sup35Δ* [*pin*$^-$] *S. cerevisiae* and *S. bayanus*

S. cerevisiae strains containing $Sup35_{SC}$, $Sup35_{SP}$ or $Sup35_{SB}$ expressed from a plasmid were transformed with each of six possible [*PSI*$^+$] inducers: $Sup35NM_{SC}$, $Sup35NM_{SC}$-HPR6.6, $Sup35NM_{SP}$-HPR6.6, $Sup35NM_{SB}$-HPR6.6, $Sup35NM_{SP}$-GFP (green fluorescent protein), and $Sup35NM_{SB}$-GFP (Figure 9A), all expressed from copper-inducible promoters. Upon induction with copper-containing medium, the strains were checked for the presence of prion by growing them on media lacking adenine. For all three full-length genes, [*PSI*$^+$] was induced by the homologous inducer containing Sup35NM from the same species (Figure 9A). However, the induction for $Sup35_{SP}$ with $Sup35NM_{SP}$-HPR6.6 was much lower than the induction for the other two homologous combinations. Interestingly, $Sup35_{SC}$ could be induced into [*PSI*$^+$] by $Sup35NM_{SP}$-HPR6.6 and $Sup35NM_{SB}$-HPR6.6 as well (Figure 9A). Heterologous induction was not observed in any other combination. In addition, Sup35NM only or Sup35NM fusions with a different extension (e.g. GFP) could not induce [*PSI*$^+$] in the [*pin*$^-$] *S. cerevisiae* cells.

S. bayanus strains containing $Sup35_{SC}$, $Sup35_{SP}$ or $Sup35_{SB}$ expressed from a plasmid were also used for induction experiments. This time, only $Sup35NM_{SC}$-HPR6.6 $Sup35NM_{SP}$-HPR6.6 and $Sup35NM_{SB}$-HPR6.6 were used as inducers. Moreover, a control group contained the three full-length proteins in strains without any expressed inducers (Figure 9B). Unlike in *S. cerevisiae*, no prion induction was observed in *S. bayanus*.

To determine whether the lack of induction in *S. bayanus* was caused by lack of expression of the inducers, the protein levels of the inducers were detected with Western blot. In *S. cerevisiae* cells where induction occurs, the expression of $Sup35NM_{SC}$-HPR6.6 inducer increased significantly after incubation on media containing 25μ*M* copper as compared to copper-free media and the full-length Sup3 protein (Figure 10). The increase between 25 and 100μ*M* was not as big as between 0 and 25μ*M*. $Sup35NM_{SC}$-HPR6.6 and $Sup35NM_{SB}$-HPR6.6 were detected in *S. bayanus* after incubation with 25μ*M* of copper because higher concentrations of copper were toxic to the cells, but it could not be determined if they were overexpressed as data for full-length Sup35 was not always available (Figure 10).

5.5. [*PSI*$^+$] Induction in *S. paradoxus*

Unlike the *S. cerevisiae* and *S. bayanus* strains, the *S. paradoxus* strain available for this study did not contain chromosomal *sup35* deletion. As a result, we could not follow the above-described induction strategy. Instead, the three possible inducers were used only on the full-length $Sup35_{SP}$ expressed from the chromosomal gene. The full-length protein showed induction resembling the one exhibited by the full-length $Sup35_{SC}$

in the *S. cerevisiae* cell environment: induction was possible with both the $Sup35NM_{SC}$-HPR6.6 and $Sup35NM_{SP}$-HPR6.6 construct. However, in *S. paradoxus*, induction was not possible with the $Sup35NM_{SB}$-HPR6.6 inducer (Figure 11). Interestingly, it seems that the heterologous $Sup35NM_{SC}$-HPR6.6 induced the [PSI^+] conversion of $Sup35_{SP}$ better that the homologous $Sup35NM_{SP}$-HPR6.6 inducer.

The phenotypic detection of [PSI^+] by growth on media lacking adenine provides only an indirect support for the presence of prions inside cells. Thus, we directly checked whether prions were seen in cells by looking at protein aggregation patterns in Ade^- *S. paradoxus* strains before induction and in an Ade^+ strain after induction and loss of the plasmid expressing the $Sup35NM_{SP}$-HPR6.6 inducer. While Sup35 proteins were not detected in the pellet of Ade^- cells, a large proportion of Sup35 was pelleted in Ade^+ cells (Figure 12), confirming the presence of prions in *S. paradoxus*.

5.6. Role of the Cell Environment in the Stability and Strength of Prions

After induction, the plasmids containing the Sup35NM-HPR6.6 fusion constructs were lost by the cells after growth on non-selective media. Then, the stability, curability and strength of the putative prions were assessed. Curability generally correlated very well with stability: stable [PSI^+] colonies were very curable by guanidine hydrochloride (data not shown). Unstable [PSI^+] were also curable, but not with as high efficiency as the stable prion variants.

The cell environment appeared to play a role on the stability of prions. For example, Sup35 from *S. paradoxus* formed unstable prions when induced in *S. cerevisiae*, but it formed both stable and unstable prions in its native *S. paradoxus* environment

(Table 4). Prions induced from $Sup35_{SP}$ were present in both strong and weak variants in both *S. cerevisiae* and *S. paradoxus*.

Similar comparisons could not be performed for Sup35 from *S. bayanus* because it could be induced only in *S. cerevisiae*, where it existed only as weak and unstable prion variants (Table 4).

6. DISCUSSION

6.1. Fusion of Part of the Human Progesterone Receptor 6.6 (HPR6.6) Protein to Sup35 Induces [*PSI*$^+$] without the Presence of Other Known Prions

In this study, we used a novel Sup35-fusion construct to induce the [*PSI*$^+$] prion isoform of Sup35 in *S. paradoxus* and *S. bayanus*. The construct contained part of the Human Progesterone Receptor 6.6 (HPR6.6), fused to Sup35NM domains, and expressed under a copper-inducible promoter (Figure 8). We showed that the construct successfully induced [*PSI*$^+$] in the [*pin*$^-$] *S. paradoxus* (Figures 7 and 11), but induction in *S. bayanus* was not detected (Figure 9B). The induction is specific to the addition of the HPR6.6 extension, as Sup35NM by itself or with another extension could not induce [*PSI*$^+$] in the [*pin*$^-$] strains (Figure 9A). Our study represents only the second instance when induction was possible without the presence of a second yeast prion. However, in the other case, only a short amino acid stretch was responsible for the observed [*PIN*$^+$]-independence (DERKATCH *et al.* 2000).

Since the portion of HPR6.6 used in this study is highly hydrophobic, it is possible that it can spontaneously aggregate in order to decrease its surface area exposed to the cytoplasm. If the prion domain of Sup35 is predisposed to aggregation, aggregation

and prion conversion could be much faster when it is fused to HPR6.6 than when the protein is not. The Sup35NM-HPR6.6 might form a "prion-like" state that later helps with the prion conversion of full-length Sup35 (Figure 13). In this way, the HPR6.6 extension could mimic the role of [*PIN*$^+$] for [*PSI*$^+$] induction and thus help overcome the [*PIN*$^+$] requirement.

6.2. [*PSI*$^+$] Prion Can Exist in *Saccharomyces* Species Other than *S. cerevisiae*

While prions are not a property of *S. cerevisiae* only, [*PSI*$^+$] prion has not been reported in other species within the *Saccharomyces* genera. Here, we report that [*PSI*$^+$] can exist in at least one other *Saccharomyces* species: *S. paradoxus* (Figures 11, 12). We showed that the lack of induction in *S. bayanus* was not caused by the lack of expression of the inducer (Figure 10). However, it is possible that the *ade1-14* reporter assay does not work in *S. bayanus* as in *S. cerevisiae* and *S. paradoxus*, so the lack of observed induction could be simply a lack of detection of induction. Indeed, only 5 of 22 *ade1-14* strains of *S. paradoxus* showed the nonsense suppression phenotype of the allele characteristic for [*PSI*$^+$] *S. cerevisiae*. Thus, it is very likely that the *ade1-14* alleles in the two *ade1-14* strains of *S. bayanus* are not functional in the nonsense suppression assay. This possibility can be checked, for example, by differential centrifugation assay after induction in *S. bayanus*.

Discrimination in prion formation between different species has been observed before. For example, Ure2 protein from *S. bayanus* is capable of forming the [*URE3*] prion in both *S. cerevisiae* and *S. bayanus*. In contrast, Ure2 from *S. paradoxus* cannot

adopt the prion conformation neither in *S. cerevisiae,* nor in *S. paradoxus* (BAUDIN-BAILLIEU *et al.* 2003), (TALAREK *et al.* 2005).

Our results also indicate that both the cell environment and intrinsic protein properties seem to play a role in prion formation. On one hand, Sup35 protein from *S. cerevisiae* can exist as a prion both in *S. cerevisiae* and *S. paradoxus*, indication that prion formation is something intrinsic to the protein. On the other hand, if the Sup35 protein indeed cannot be induced into a prion in *S. bayanus*, it will mean that there is something different in this species when compared to *S. cerevisiae* and *S. paradoxus*. One possible explanation is that *S. bayanus* could contain some factors that inhibit the conversion between the normal and prion isoforms of the protein. The other possibility is that there could be factors in *S. cerevisiae* and *S. paradoxus*, other than known prions, that are essential for catalyzing the prion conversion of the normal cell protein.

It is important to note that both explanations for the role of the cell environment evoke the existence of additional cell factors. The identification of these factors could be important in prion studies, especially if they elucidate the mechanism by which a normal cell protein can switch conformations and aggregate. Even more important, these factors could be conserved in humans and could have an effect in aggregate formation in the brains of patients with neurodegenerative diseases.

The cell environment also plays a role in the stability of prions. From the studies of [*PSI*$^{+}$] prion formed by the conversion of $Sup35_{SP}$ in both *S. cerevisiae* and *S. paradoxus*, it seems that prion conversion in the homologous cell environment is preferred and gives rise to more stable prion variants. Still, the successful prion induction in more species is required to confirm this generalization.

6.3. The Species Barrier in Prion Transmission Could be Controlled at the Level of Prion Induction

Prion induction in *S. bayanus* notwithstanding, the induction of the full-length Sup35 protein by a homologous inducer was always possible, but the heterologous induction was rarer. This induction outcome was not surprising because the inducers contain the N region of Sup35: the prion domain of the protein that causes the species barrier in the *Saccharomyces* genera (CHEN *et al.* 2007). Extrapolating the findings to the interactions of two full-length divergent proteins, one of them would not be able to cause the prion conversion of the other. Thus, there will be no prions that can be passed on to daughter cells, explaining the species barrier.

The rare heterologous induction can be explained if we consider that $Sup35NM_{SC}$ and $Sup35NM_{SP}$ give rise to prion aggregates with slightly different conformations. Each conformation will be more likely to seed the prion conversion of its homologous full-length protein, but heterologous conversion will not be completely impossible.

7. CONCLUSIONS

- *S. paradoxus* and *S. bayanus*, two closely-related species to *S. cerevisiae*, lack the [PIN^+] prion
- Sup35NM constructs containing part of the Human Progesterone Receptor 6.6 (HPR6.6) protein can induce *S. paradoxus* and *S. bayanus* Sup35 proteins into prions without the presence of other known endogenous prions

- *S. paradoxus* Sup35 protein can be induced into a prion state in *S. paradoxus* cells, thus demonstrating that ability to form a prion is not a unique property of *S. cerevisiae* Sup35 protein
- No prion formation by Sup35 protein was detected in *S. bayanus*
- Mitotically stable prion variants of *S. paradoxus* Sup35, not detected in the heterologous (*S. cerevisiae*) cell environment, can be generated in the homologous (*S. paradoxus*) environment

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9. APPENDIX A: FIGURES

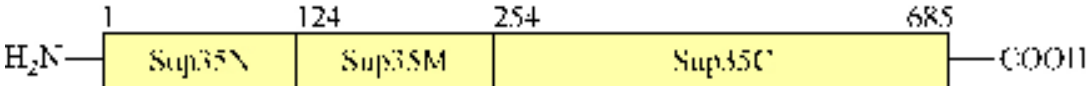


Figure 1. Structure of Sup35 protein. The prion forming domain, Sup35N, is located at the amino-terminus of the protein, while the region at the carboxy-terminus is the functional Sup35C. Sup35M is between the Sup35N and Sup35C domains. The numbers above the domains correspond to the amino acid positions where the different domains start. An exception is position 685, which is the last amino acid of the protein.

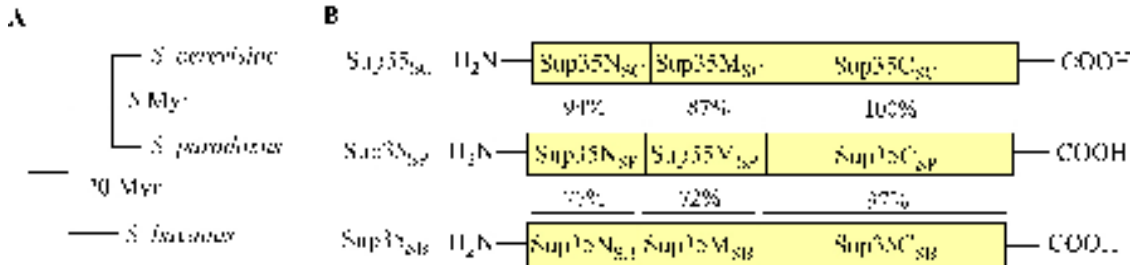


Figure 2. Experimental system. **A.** Species in the *Saccharomyces* genera used in the current study. Experiments were carried out in *S. cerevisiae*, *S. paradoxus* and *S. bayanus*. The numbers within the phylogenetic tree represent the divergence time between the different species in millions of years (Myr). **B.** Sup35 in the *Saccharomyces* genera. The overall structure of Sup35 is the same in *S. cerevisiae* ($Sup35_{SC}$), *S. paradoxus* ($Sup35_{SP}$) and *S. bayanus* ($Sup35_{SB}$). The numbers above the different regions show the amino acid sequence identity as compared to the amino acid sequence of Sup35 in *S. cerevisiae*. Sup35 from *S. bayanus* is missing nine amino acids, so the protein is a little shorter than the *S. cerevisiae* and *S. paradoxus* proteins.

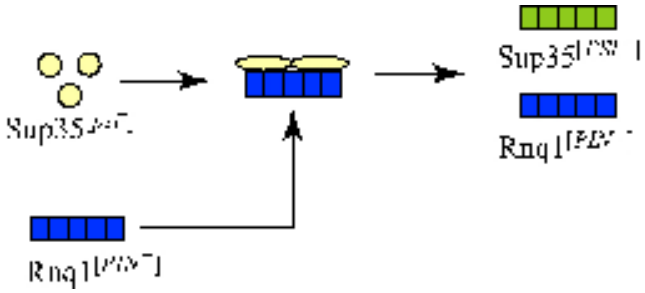


Figure 3. A model for the role of [*PIN*$^+$] prion in [*PSI*$^+$] induction. When Rnq1 protein is in its aggregated, [*PIN*$^+$] prion isoform, it could act as a scaffold where Sup35 can bind and undergo the conformational change required for its conversion from non-prion, [*psi*$^-$], isoform to prion [*PSI*$^+$] isoform. After the prion conversion of Sup35, the [*PSI*$^+$] aggregates and the Sup35NM-HPR6.6 aggregates most likely separate.

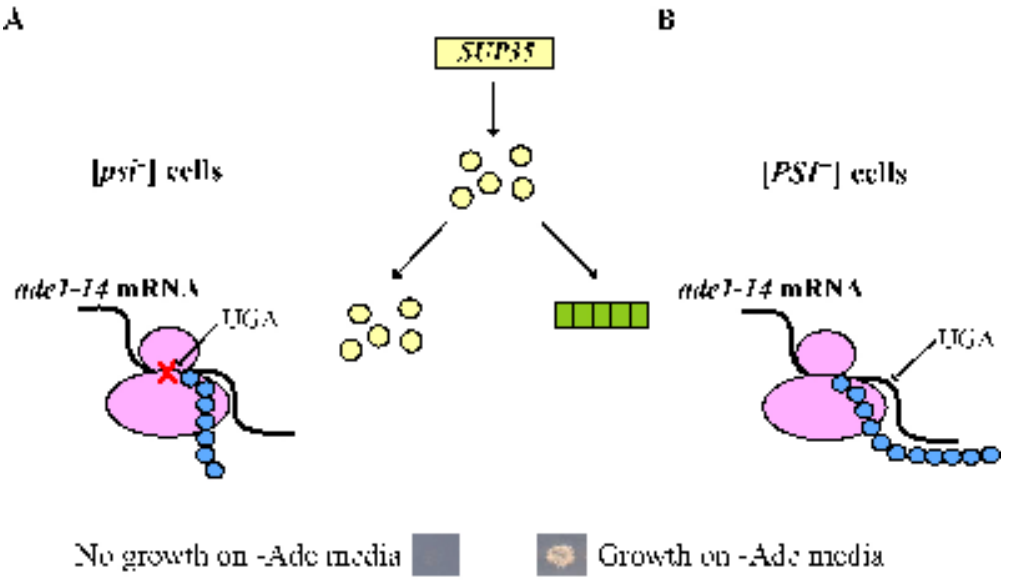


Figure 4. Nonsense suppression assay used to detect [*PSI*$^+$] prion. The cells contain a reporter *ade1-14* allele with a premature UGA stop codon in the *ADE1* gene. **A.** In prion-free, [*psi*$^-$], cells, Sup35 is completely functional, leading to a truncated Ade1 protein, and making cells unable to grow on media lacking adenine (-Ade media). **B.** In [*PSI*$^+$] cells, Sup35 is in an aggregated, partially inactive state, the stop codon is not recognized, and the full-length Ade1 is produced. Cells can grow on media lacking adenine.

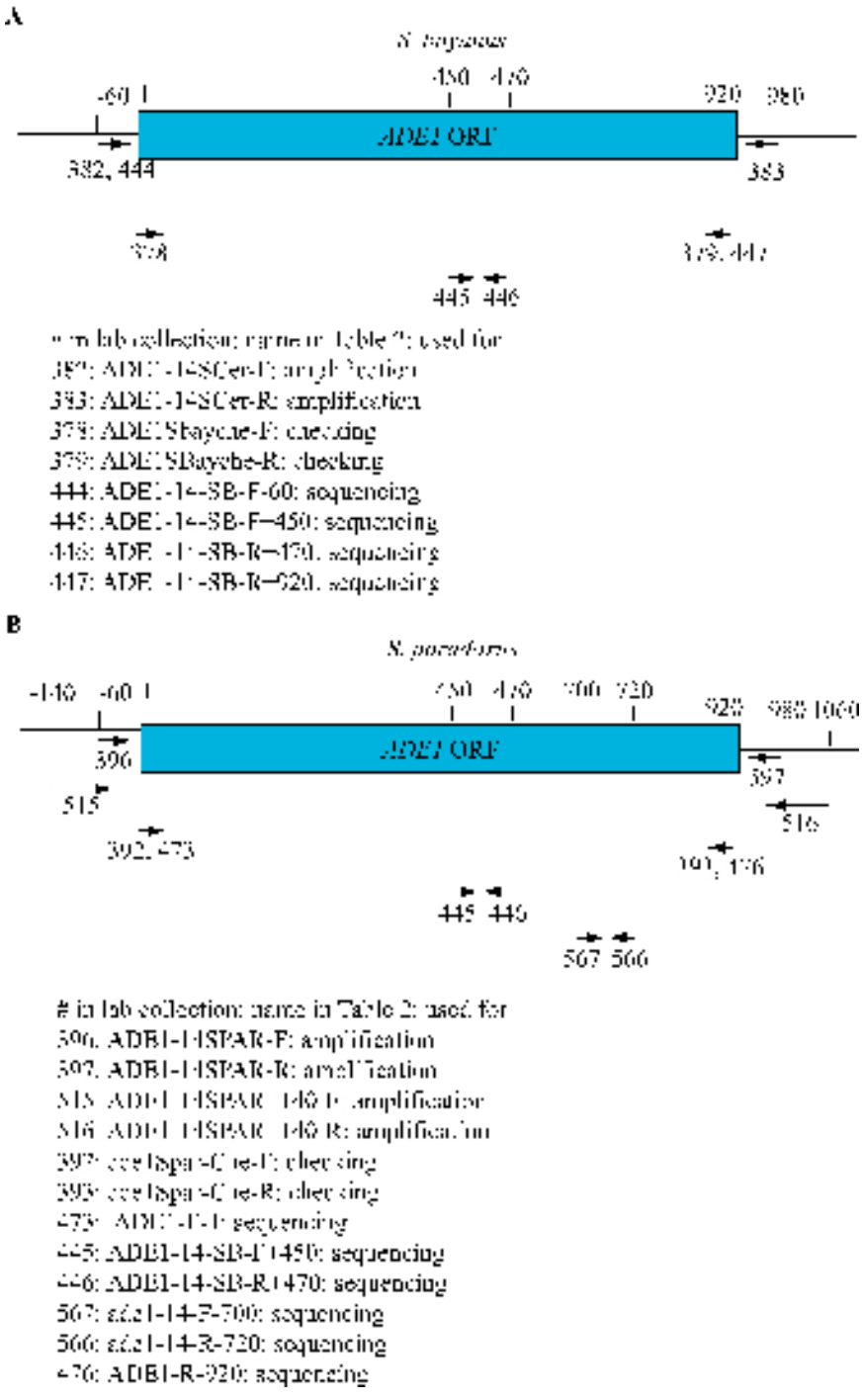
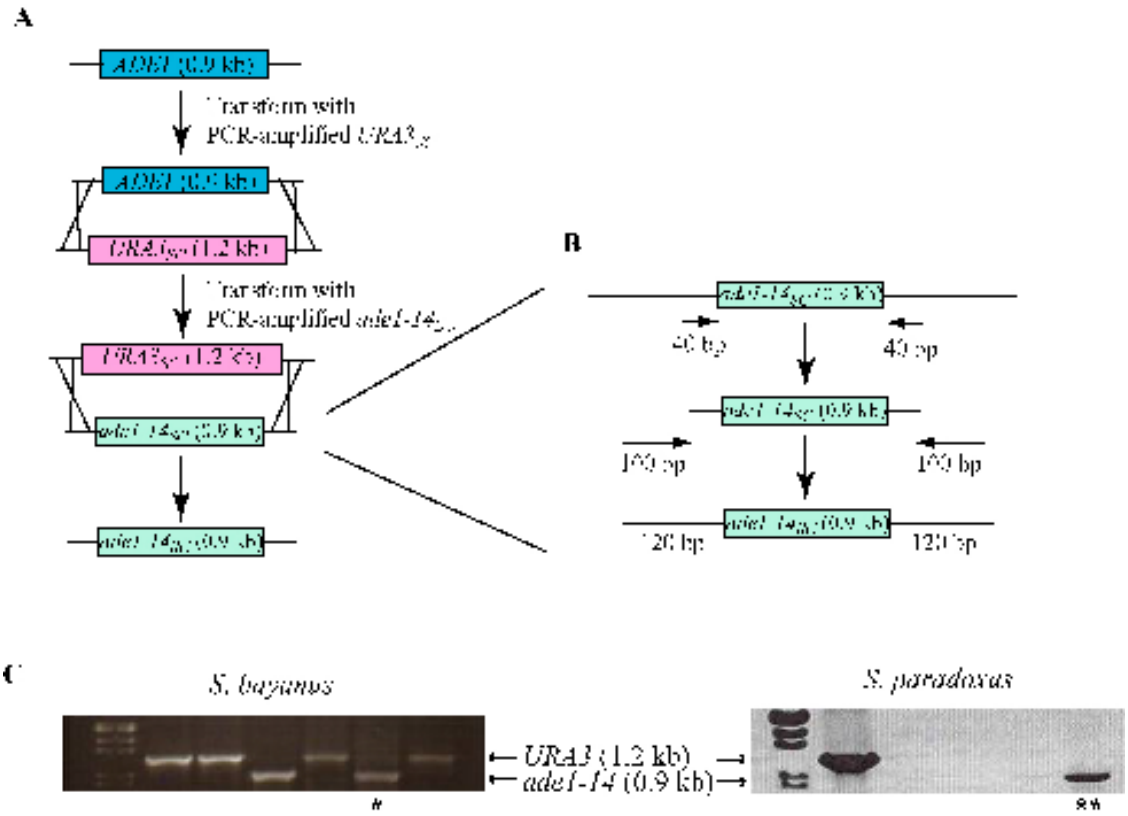


Figure 5. Binding sites for primers associated with *ade1-14* strain creation for *S. bayanus* (**A**) and *S. paradoxus* (**B**). The arrows show the direction of the primers. The numbers above *ADE1* correspond to the binding positions of the primers. Not all primers bind directly to the chromosomal *ADE1*. See Table 2 and text for detail descriptions of the primers.



D

```
   1 ATGTCAATCA CGAAGACTGA ACTCGACCGC TATATGCCAT TGGTGCCCAG
  51 AGGTAAAGTT AGAGACATTA TATCAGGTAA CCCTGGTACG TTGCTGTTTG
 101 CTGCTACCGA TCCTATCTCT GCATATGACG TTATTATCGA AAACAGCATT
 151 CCTGAAAAGG GGATCCATCT CGACAAACTG TCAGACTTCT GGTTCAAGTT
 201 CCTGTCCAAC GATGTTCGTA ATCATTCGCT CGACATCGCC CCAGGTAAGA
 251 CTATTTCGAT TATCTACCTG GCAAAATTGA GCGAACCAAA GTACAAAACG
 301 CAACTAGAAG ACCCCCTCTC TATTGCTCCA AAACATAAAT TAACTCCACT
 351 GGAAGTAAAT TGTCAGAGCT ACATCACCGG TTCTGCTTCG AAAGAGTACG
 401 TAAAAACAGG TACTCTGCAT GCTCTGAAAC AACCTAAGGA CTTAAAGAA
 451 TCTCAAGAGT TCCCAGAACC AATCTTCACC CCATCGACCA AGGCTGAACA
 501 AGGTCAAATA GACGAAAATA TCTCTCCTGC CCAGCCCGCT CAGCAGGTGG
 551 GTCAAGATTT GTCACGTAGA GTGGCAGAAC TGGGTGTAAA ACTGTACTCC
 601 AAGTCCAAAG ATTATGCTAA GGAGAACGCC ATCATCATCG CAGACATTAA
 651 ATTCGATTTC GGTATTGACG AAAAGACCAA TCAAATTATT CTAGTGGACG
 701 AGGTCCTAAC GCCAGATTCC TCTTAGATCT TGAAACCGTG CTCTTATAAC
 751 GTAGGAGAAT CCCAAGATTC TTTACGATAA CCAATTTTAA GACACTGCT
 801 TACTGCTAAA AAGTTGAACC CTCTTAACGa aaTCAAAATG CCCCAAGACA
 851 TTGTTCGACA GTACAAGCGC AAATATATAG AGCGCTTATG AACATTGACA
 901 GGGTCTAAAT GGTCTCACTA A
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Figure 6. Introduction of the *ade1-14* reporter allele in *S. paradoxus* and *S. bayanus*. **A.** Approach for the replacement of *ADE1* in *S. bayanus* with *ade1-14* from *S. cerevisiae*. *ADE1* (approximate size of 0.9kb) in *S paradoxus* and *S. bayanus* was first replaced by a PCR-amplified copy of *URA3* (approximate size of

1.2kb) from *S. cerevisiae* by transformation. Then, *URA3* was replaced with the PCR-amplified *ade1-14* from *S. cerevisiae* by a second transformation. All genes were amplified with 40bp outside of the open reading frame (ORF) to facilitate homologous recombination. **B.** Modified approach for *ade1-14* introduction in *S. paradoxus*. The basic sequence of steps was the same as the replacement in *S. bayanus*. However, after *ade1-14* was amplified from *S. cerevisiae* with 40bp outside of the ORF, a second set of primers was used to introduce longer homologous region to *S. paradoxus*. The second set of primers was 100bp long, with 20bp overlapping with the original primers. The other 80bp contained sequence homologous to the promoter and terminator region of *ADE1* in *S. paradoxus*. **C.** Potential replacements of *URA3* with *ade1-14* for both *S. bayanus* and *S. paradoxus* were checked on a 1% agarose gel. (*) marks a potential in *S. bayanus* which proved to be a correct replacement after sequencing. (**) is a potential in S. paradoxus that contains the *ade1-14* allele. However, it contains two amino acid substitutions (accidentally introduced by PCR) when compared to the sequence of *ade1-14* from *S. cerevisiae*. **D.** DNA sequence of the *S. paradoxus* (**) potential. The premature stop codon is indicated by red capital letters. The substitutions compared to *ade1-14* from *S. cerevisiae* are marked in lower-case red letter. Both substitutions are G→A, and lead to the amino acid changes G277D and V278I.

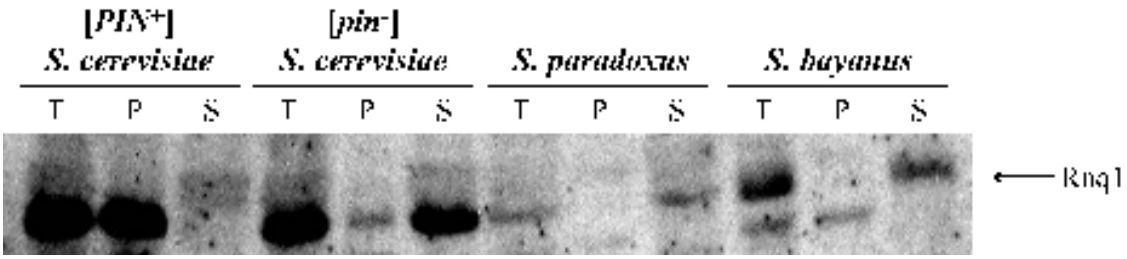

Figure 7. [*PIN*$^{+}$] status of *S. paradoxus* and *S. bayanus*. Total proteins were isolated from *S. cerevisiae* control [*PIN*$^{+}$] and [*pin*$^{-}$] strains, and from *S. paradoxus* and *S. bayanus* after growing the strains overnight in liquid YPD. The total lysate (T) was centrifuged at 53,000 *g* for 30 minutes to obtain supernatant (S) and pellet (P). Each fraction was boiled in SDS, run on an 8% polyacrylamide gel, and transferred to a HyBond^{TM} ECL^{TM} nitrocellulose membrane. The proteins were detected with a rabbit primary antibody to Rnq1 (1:5000 v/v dilution) and a rabbit HRP secondary antibody (1:3000 v/v dilution).

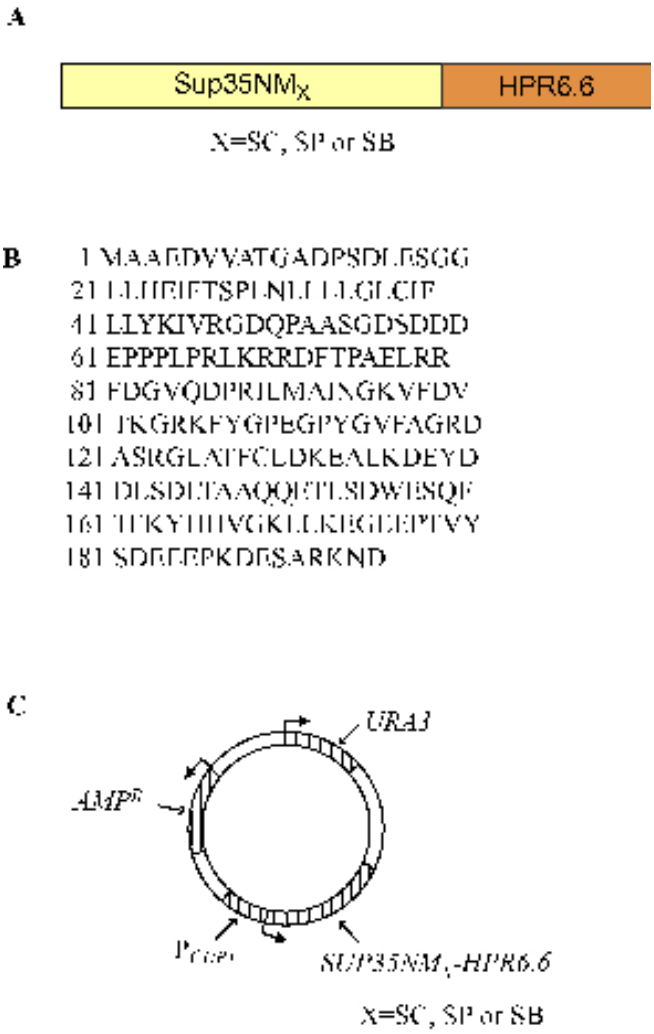

Figure 8. HPR6.6-fusion constructs used for [*PSI*$^+$] induction. **A.** Sup35NM from *S. cerevisiae*, *S. paradoxus* and *S. bayanus* were each fused to a part of the Human Progesterone Receptor 6.6 protein (HPR6.6). **B.** Actual amino acid sequence (one-letter code) of HPR6.6 that was used in the fusion constructs. **C.** A simplified scheme for the shuffle plasmids used for the expression of the *SUP35NM-HPR6.6* constructs. The plasmids contained the *SUP35NM-HPR6.6* fusions under the control of the inducible *CUP1* promoter (P_{CUP1}). *URA3* and ampicillin-resistance (AMP^R) genes were the selectable markers for yeast and E. coli, respectively.

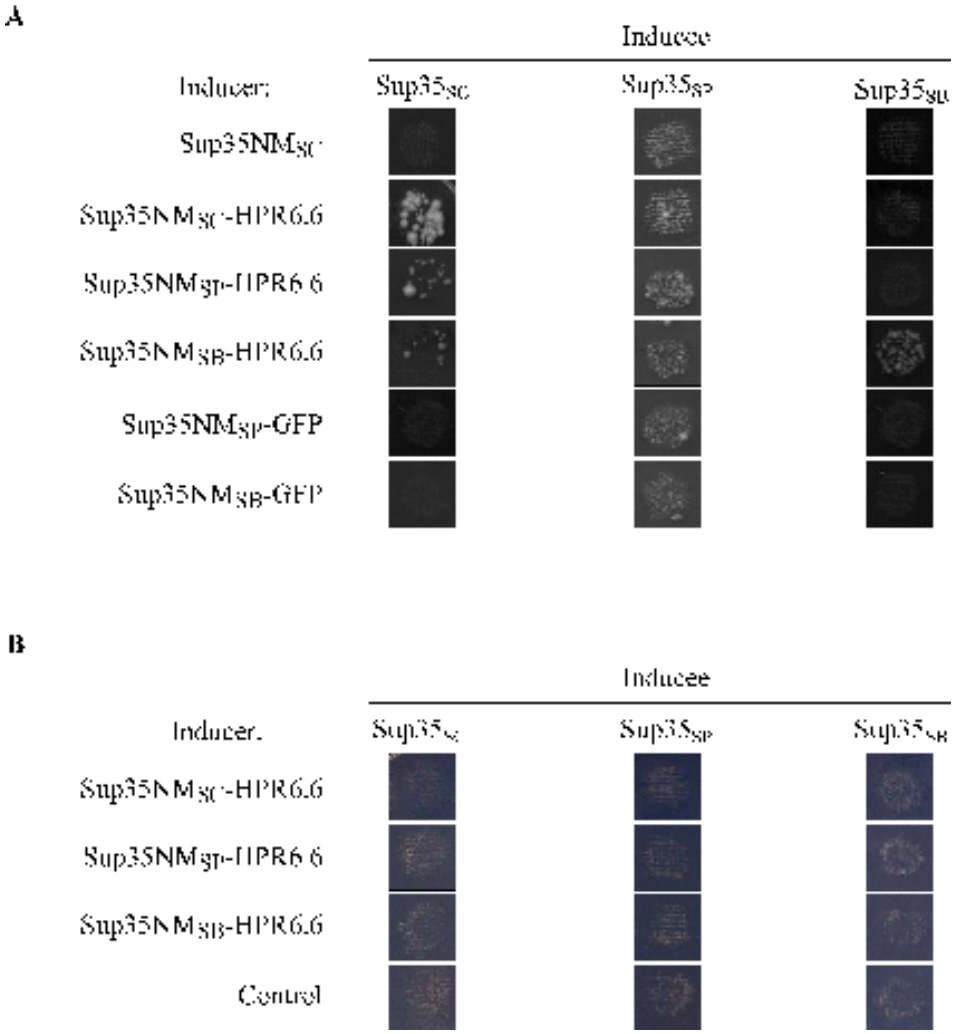

Figure 9. [*PSI*$^+$] induction in *S. cerevisiae* and *S. bayanus*. **A.** *S. cerevisiae* strains containing $Sup35_{SC}$, $Sup35_{SP}$ or $Sup35_{SB}$ expressed from a plasmid with endogenous *SUP35* promoter were induced to become [*PSI*$^+$] by the overexpression of Sup35NM, $Sup35NM_{SC}$-HPR6.6, $Sup35NM_{SP}$-HPR6.6, $Sup35NM_{SB}$-HPR6.6, $Sup35NM_{SP}$-GFP, or $Sup35NM_{SB}$-GFP from the *CUP1* copper-inducible promoter. Approximately 12 transformants for each combination were grown on media containing 100μM $CuSO_4$ for two days and replica-plated to media lacking adenine. The pictures show representative transformants from media lacking adenine after growth with 100μM $CuSO_4$ for 15 days. **B.** *S. bayanus* strains containing $Sup35_{SC}$, $Sup35_{SP}$ or $Sup35_{SB}$ expressed from a plasmid with endogenous *SUP35* promoter were induced to become [*PSI*$^+$] by the overexpression of $Sup35NM_{SC}$-HPR6.6, $Sup35NM_{SP}$-HPR6.6 or $Sup35NM_{SB}$-HPR6.6 from the *CUP1* copper-inducible promoter. A control experiment contained no inducer.

Approximately 12 transformants were grown on media containing 25μM $CuSO_4$ for one day and replica-plated to media lacking adenine. The pictures show representative transformants from media lacking adenine after growth with 25μM $CuSO_4$ after 21 days.

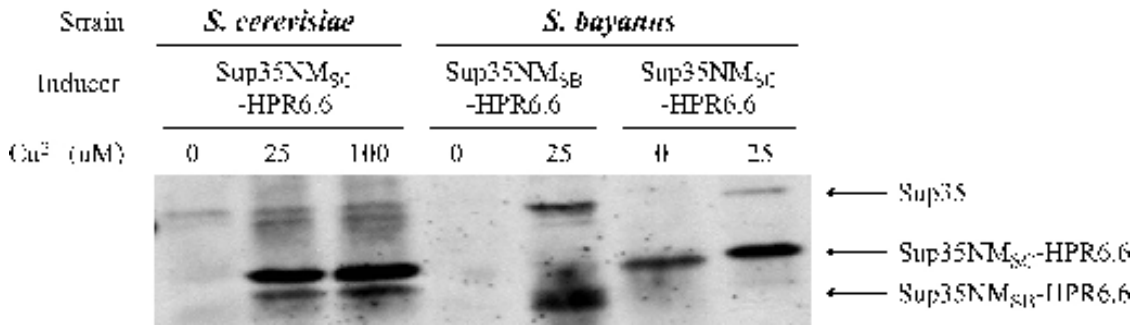

Figure 10. Expression of Sup35 and Sup35NM$_{SC}$-HPR6.6 and Sup35NM$_{SB}$-HPR6.6 inducers in *S. cerevisiae* and *S. bayanus*. The Sup35NM$_{SC}$-HPR6.6 and Sup35NM$_{SB}$-HPR6.6 inducers were expressed from the *CUP1* copper-inducible promoter, while full-length Sup35 was expressed from its own *SUP35* promoter. Expression levels were checked by Western blot after strains containing the inducers and the full-length proteins were grown on media containing 0, 25 or 100μM $CuSO_4$ for *S. cerevisiae*, and 0 and 25μM $CuSO_4$ for *S. bayanus*. The proteins were detected with a rabbit primary antibody to Sup35NM (1:2000 v/v dilution), and a rabbit HRP secondary antibody (1:2000 v/v dilution).

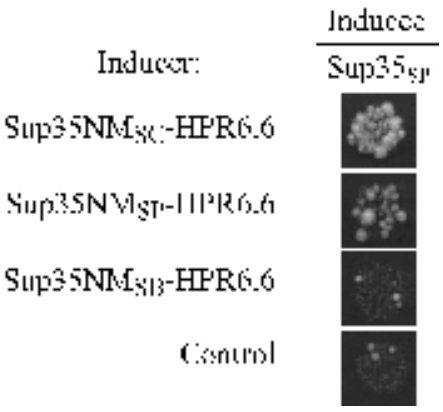

Figure 11. [*PSI*$^+$] induction in *S. paradoxus*. The wild-type chromosomal Sup35 from *S. paradoxus* was induced to become [*PSI*$^+$] by the overexpression of Sup35NM$_{SC}$-HPR6.6, Sup35NM$_{SP}$-HPR6.6 or Sup35NM$_{SB}$-HPR6.6 from the *CUP1* copper-inducible promoter. A control experiment contained no inducer. Approximately 12 transformants were grown on media containing 25μM $CuSO_4$ for one day and replica-plated to media lacking adenine. The pictures show representative transformants from media lacking adenine after growth with 25μM $CuSO_4$ for 21 days.

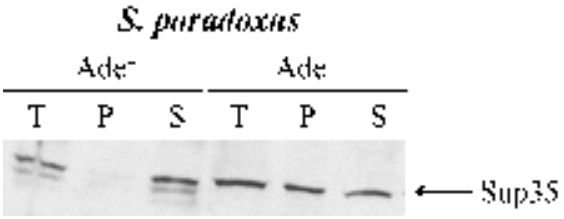

Figure 12. Sup35 aggregation in *S. paradoxus*. *S. paradoxus* strains before induction (Ade^-), and after induction and loss of the inducing plasmid for a single Ade^+ colony were each grown overnight in liquid YPD. The total proteins were isolated to obtain the total cell lysate (T). The total lysate was centrifuged at 10,000 *g* for 15 minutes to separate the supernatant (S) and pellet (P). Each fraction was boiled in SDS, run on an 8% polyacrylamide gel, and transferred to a HyBond™ ECL™ nitrocellulose membrane. The proteins were detected with a rabbit primary antibody to Sup35NM (1:2000 v/v dilution) and a rabbit HRP secondary antibody (1:2000 v/v dilution).

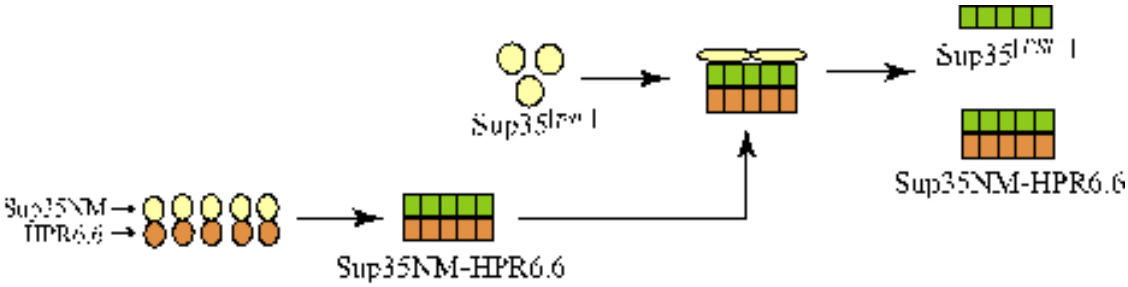

Figure 13. A model for the role of the Sup35NM-HPR6.6 fusion constructs in [*PSI*$^+$] induction. The overexpression of Sup35NM-HPR6.6 and potentially the hydrophobicity of HPR6.6 itself could make the fusion constructs likely to aggregate in a prion-like state. This aggregated state could then act as a scaffold where Sup35 can bind and undergo the conformational change required for its conversion from non-prion, [*psi*$^-$], isoform to prion [*PSI*$^+$] isoform. After the prion conversion of Sup35, the [*PSI*$^+$] aggregates and the Sup35NM-HPR6.6 aggregates most likely separate.

10. APPENDIX B: TABLES

Table 1. List of Yeast Strains Used in This Study

Strain Name*	Genotype	Used for
	***Saccharomyces cerevisiae* strains**	
GT671	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN LEU2 SUP35*$_{SC}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT797	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN URA3 SUP35*$_{SP}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT811	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN URA3 SUP35*$_{SC}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT826	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN LEU2 SUP35*$_{SP}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT883	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN LEU2 SUP35*$_{SB}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT987	*MATα ade1-14 his3Δ (or 11,15) lys2 ura3-2 leu3,112 trp1 sup35::HIS3* [*CEN URA3 SUP35*$_{SB}$] [*psi*$^-$][*pin*$^-$]	Prion induction
	***Saccharomyces paradoxus* strains**	
GT983-2A	*MATa ura3-P2 lys2 Δho::KanMX6*	Strain creation
GT992	*MATa ura3-P2 lys2 Δho::KanMX6 ade1*$_{SP}$*::URA3*$_{SC}$ [*psi*$^-$][*pin*$^-$]	Strain creation
GT1142	*MATa ura3-P2 lys2 Δho::KanMX6 ade1*$_{SP}$*::ade1-14*$_{SC}$*(G277N, V278I)*[*psi*$^-$][*pin*$^-$]	Prion induction
	***Saccharomyces bayanus* strains**	
Su1A	*MATa ura3-1 ho::KanMX4* [*psi*$^-$][*pin*$^-$]	Strain creation
GT986	*MATa ura3-1 ade1::URA3*$_{SC}$ *ho::KanMX4* [*psi*$^-$][*pin*$^-$]	Strain creation
GT1020	*MATα ura3-1 lys2 ho::KanMX4* [*psi*$^-$][*pin*$^-$]	Strain creation
GT1028	*MATa ura3-1 ade1::ade1-14*$_{SC}$ *ho::KanMX4* [*psi*$^-$][*pin*$^-$]	Strain creation
GT1131	*MATα ura3-1 ade1::ade1-14*$_{SC}$ *sup35Δ::ClonNAT ho::KanMX4* [*CEN LYS2 SUP35*$_{SP}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT1132	*MATα ura3-1 ade1::ade1-14*$_{SC}$ *sup35Δ::ClonNAT ho::KanMX4* [*CEN LYS2 SUP35*$_{SB}$] [*psi*$^-$][*pin*$^-$]	Prion induction
GT1133	*MATα ura3-1 ade1::ade1-14*$_{SC}$ *sup35Δ::ClonNAT ho::KanMX4* [*CEN LYS2 SUP35*$_{SC}$] [*psi*$^-$][*pin*$^-$]	Prion induction

* All strains except Su1A were created for this study. Su1A was created by Talarek *et al.* (2004)

Table 2. List of Primers Used in This Study

Primer Name	Number*	Sequence	Used for
Associated with *S. bayanus* strain creation			
ADE1-14 SCer-F**	382	5' CTGGGCCAAC CGCATCGGAA GCACTGCTTA GAGGGATATC ATACAAAGAG AGAAGCAAGA ATGTCAATTA CGAAGACTGA 3'	Forward for amplifying *ade1-14*$_{SC}$ with 60 bp upstream homologous to *ADE1*$_{SB}$ promoter
ADE1-14 SCer-R	383	5' TACGTATGTA TATATTTAGT GCGAGATTCA CTGATGACCT GTAACAAATA GAAAGAACGC TTAGTGAGAC CATTTAGACC 3'	Reverse for amplifying *ade1-14*$_{SC}$ with 60 bp downstream homol. to *ADE1*$_{SB}$ terminator
ADE1Sbay che-F	378	5' ATACAAAGAG AGAAGCAAGA 3'	Forward for checking *ade1-14* potentials
ADE1Sbay che-R	379	5' GTAACAAATAG AAAGAACGC 3'	Reverse for checking *ade1-14* potentials
ADE1-14 SB-F-60	444	5' CTGGGCCAAC CGCATCGGAA 3'	Forward for sequencing *ade1-14*, starts 60 bp upstream of ORF
ADE1-14-SB-F+450	445	5' TCTCAAGAGT TCCCAGAACC 3'	Forward for sequencing *ade1-14,* starts at 450 bp of ORF
ADE1-14-SB-R+470	446	5' GGTTCTGGGA ACTCTTGAGA 3'	Reverse for sequencing *ade1-14,* starts at 470 bp of ORF
ADE1-14-SB-R+980	447	5' TACGTATGTA TATATTTAGT 3'	Reverse for sequencing *ade1-14,* starts at ~60 bp after ORF
sup35SB:: natNT2-F	535	5' GTTTACTAGC AACAGTACCT ATACCTGCCC ACTAGTAATC GGATCCCCGG GTTAATTAAG 3'	Amplifying *ClonNAT* gene, with 40 bp homol. to *SUP35*$_{SB}$ prom.
sup35SB:: natNT2-R	536	5' TGGGGTTGTT TTTTTTTTTC GTTTAATTCT TGCGAAAAA AGAGCTCGAT TACAACAGGTG 3'	Amplifying *ClonNAT* gene, with 40 bp homol. to *SUP35*$_{SB}$ term.
sup35SB:: natNT2-check-F	534	5' ATACCTGCCCA CTAGCAATC 3'	Forward for checking *sup35Δ* potentials
sup35SB:: natNT2-check-R	533	5' GTTTAATTCT TGCGAAAAAA 3'	Reverse for checking *sup35Δ* potentials
Associated with *S. paradoxus* strain creation			
ADE1-14 SPAR-F	396	5' AGAATCAATT GAATCATAAG CATTACTTAT AAAGAATACA CATACGAAAA GTAATAACAA TGTCAATTAC GAAGACTGA 3'	Forward for amplifying *ade1-14*$_{SC}$ with 60 bp upstream homologous to *ADE1*$_{SP}$ promoter
ADE1-14 SPAR-R	397	5' ATGTATGATT CATATTTAGT GCGAAGTACA CTGGCGACTT GTAGCATATG TAAAAACACT TTAGTGAGAC CATTTAGACC 3'	Reverse for amplifying *ade1-14*$_{SC}$ with 60 bp downstream homol. to *ADE1*$_{SP}$ terminator

ADE1-14SPAR +140-F	515	5’ TATATATATAT GTACATTCTC ACCTGGATTC TTTGGGGGTA AAACGGTTGA GTGTTGTGCT TTTGTAGTTG GTACTGTTAA GAATCAATTG AATCATAA G 3’	Forward for amplifying *ade1-14$_{SC}$* with 140 bp upstream homol. to *ADE1$_{SP}$* promoter. Last 20 bp overlap with ADE1-14SPAR-F
ADE1-14SPAR +140-R	516	5’ CGCCAAACCT GCATACCACT GGCAAACAAG ATATCGATAA GACTTGCTTT GAGAACATTT ATACATTAAT ACATATGGGT ATGTATGATT CATATTTAG T 3’	Reverse for amplifying *ade1-14$_{SC}$* with 140 bp downstream hom. to *ADE1$_{SP}$* terminator. Last 20 bp overlap with ADE1-14SPAR-R
ade1Spar-Che-F	392	5’ CATACGAAAA AGTAATAACA 3’	Forward for checking *ade1-14* potentials
ade1Spar-Che-R	393	5’ GTAGCATATG TAAAAACACT 3’	Reverse for checking *ade1-14* potentials
ADE1-F-1	473	5’ ATGTCAATTA CGAAGACTGA 3’	Forward for sequencing *ade1-14*, starts at position 1 of ORF
ADE1-R-920	476	5’ TAGTGAGACC ATTTAGACCC 3’	Reverse for sequencing *ade1-14*, starts position 920 of ORF
ADE1-14-SB-F+450	445	5’ TCTCAAGAGT TCCCAGAACC 3’	Forward for sequencing *ade1-14,* starts at 450 bp of ORF
ADE1-14-SB-R+470	446	5’ GGTTCTGGGA ACTCTTGAGA 3’	Reverse for sequencing *ade1-14,* starts at 470 bp of ORF
ade1-14-F -700	567	5’ AGGTGCTAAC GCCAGACTCC 3’	Forward for sequencing *ade1-14*, starts at position 700 of ORF
ade1-14-R -720	566	5’ GGAGTCTGGC GTTAGCACCT 3’	Reverse for sequencing *ade1-14*, starts position 720 of ORF

* Number of primer as entered in lab collection

** See Figure 5 for graphical representation of where the different primers associated with *ADE1/ade1-14* bind

Table 3. List of Plasmids Used in This Study

Plasmid Name*	Promoter	*SUP35* gene	Yeast Marker
pYCL-CUP-NMSc	P_{CUP1}	$SUP35NM_{SC}$	*LEU2*
pYCL-CUP-NMScHPR6.6	P_{CUP1}	$SUP35NM_{SC}$	*LEU2*
PmCUPs-Sup35NMSpar-HPR6.6	P_{CUP1}	$SUP35NM_{SP}$	*URA3*
PmCUPs-Sup35NMSpar-sGFP#2	P_{CUP1}	$SUP35NM_{SP}$	*URA3*
PmCUPs-Sup35NMSbay-HPR6.6	P_{CUP1}	$SUP35NM_{SB}$	*URA3*
PmCUPs-Sup35NMSbay-sGFP#2	P_{CUP1}	$SUP35NM_{SB}$	*URA3*
pRS316-CUP-SUP35NM-HPR6.6	P_{CUP1}	$SUP35NM_{SC}$	*URA3*
pRS316-GAL**	P_{GAL1}	None	*URA3*

* All plasmids are centromeric
** Reference: (HARRIS and TRUE 2006). All other plasmids were created for this study

Table 4. Stability and strength of the induced prions in different cell environments

Cell environment	Sup35 protein	Induction possible by			Stability	Suppression strength
		$Sup35NM_{SC}$*	**$Sup35NM_{SP}$***	**$Sup35NM_{SB}$***		
S. cerevisiae	$Sup35_{SC}$	Yes	Yes	No	Stable and unstable	Strong and weak
	$Sup35_{SP}$	No	Yes	No	Unstable	Strong and weak
	$Sup35_{SB}$	No	No	Yes	Unstable	Weak
S. paradoxus	$Sup35_{SP}$	Yes	Yes	No	Stable and unstable	Strong and weak
S. bayanus	$Sup35_{SC}$	No	No	No	N/A	N/A
	$Sup35_{SP}$	No	No	No	N/A	N/A
	$Sup35_{SB}$	No	No	No	N/A	N/A

* The inducers are the HPR6.6-fusion constructs of Sup35NM